SURFACE MODIFICATION FOR BIOCOMPATIBILITY

Fourth Quarterly Report
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FIGURE CAPTIONS

Figure 1   Effects of plating density on the cell viability in culture. Phase-contrast photomicrographs of cortical cells seeded at $50 \times 10^3/cm^2$ (1 and 2), and $100 \times 10^3/cm^2$ (3 and 4) in serum-free medium for 24 hours (1 and 3) and 7 days (2 and 4). The cells are from 16-day-old rat embryos and plated on poly-D-lysine. Higher plating density can increases survival or cortical cells.

Figure 2   Effects of plating density on the cell viability in serum-free medium with CNTF. Phase-contrast photomicrographs of cortical cells seeded at $50 \times 10^3/cm^2$ (1 and 2), and $100 \times 10^3/cm^2$ (3 and 4) for 24 hours (1 and 3) and 7 days (2 and 4). The cells are from 16-day-old rat embryos and plated on poly-D-lysine. Cells plated at higher density with addition of CNTF suffer less mortality.

Figure 3   Serum-free Neurobasal/B27 medium supports longer-term growth of cells from E16 embryonic rat cerebral cortex. Phase-contrast photomicrographs of cortical cells cultured in Neurobasal (NB) only (1 and 4), NB with N3 (2 and 5) and NB with B27 (3 and 6) for 5 (1-3) and 11 (4-6) days. Cells in NB+N3 show better survival and growth at day 5 in culture than those in NB+B27. At Day 7, cells in NB+B27 show better growth than in NB+N3. Both NB+N3 and NB+B27 support cell growth better than NB only.

Figure 4   Neuronal and astrocytic expression of embryonic cortical cells cultured in serum-free medium on poly-D-lysine and DETA for 21 days. Fluorescence photomicrographs (top and middle panels) of NSE (neuron-specific enolase)- and GFAP- immunoreactive cells cultured from E16 rat cerebral cortex. Cells were incubated simultaneously with rabbit anti-NSE and mouse anti-GFAP, followed by incubation with a mixture of fluorescein-conjugated goat anti-mouse IgG and rodamine-conjugated goat anti-rabbit IgG. Most cells are stained by anti-GFAP (green). About 5% of total cells express NES (red) on poly-D-lysine and DETA. Clusters of NES+ cells are often seen on DETA. In the bottom panel are phase-contrast photomicrographs showing cortical cells cultured on poly-D-lysine in serum-free medium with and without CNTF.

TABLE CAPTIONS

Table 1   Long term stability experiments of silanes with and without added BSA.

PROJECT SUMMARY FOR FOURTH QUARTER

The aim of this work is to create surfaces on implantable silicon microstructures for the purpose of controlling the interaction of neurons, glia, and related cells and their protein products with the microstructure. As presented in the third quarterly report, we established a rudimentary serum-free culture to approximate the composition of cerebral spinal fluid (CSF), and have screened thirteen (13) artificial surfaces for E19 cortical cell response; 5 artificial for E22 cortical cell response; and 5 artificial surfaces for glial cell response. We have begun screening E16 cortical cells to extend longevity. Survival was extended to 21 days. After discussion, we determined it was in the best interest of the project to focus on the key issue, longevity in culture, that has impact on all other stated objectives. We need to develop a significant understanding of how culture conditions specifically affect cell survival, growth and population dynamics, over time, of not only what is essentially a purified neuronal culture but also how the manipulation of conditional influences affects other cell types. This is critical toward application of successful implantation and utilization of prostheses *in vivo*. As a result, this fourth quarter was primarily devoted to improving the long term viability and longevity of the serum-free *in vitro* culture protocol, and examining the role that conditions can determine cell type, distribution and neuronal neurotransmitter expression utilizing immunohistochemistry. We reprioritized our fourth quarter objectives with regard to experiments with E22 and PN 10 cultures and included them in the next quarter's objectives.

Techniques and training for production of microglial cultures was astutely provided by Dr. Carol Coulton. However, her group's cultures are most successful with hamster tissue. We are now extending the conditions to determine our own methods to maximize long term survival of rat microglia.

Continuing surface stability experiments indicate that at least some of the artificial surfaces (silane monolayers on glass) are stable for periods of up to 12 weeks in the presence of proteins. We are extending these experiments to other surfaces, but must now address the problem that in the presence of adsorbing protein overlayers, the XPS conditions on some of the artificial surfaces are obscured.

We have recently hired a surface chemist, Dr. Thomas Schneider. He will come on board February 12. His expertise will lend itself toward production of novel and effective biologically modified SAM surfaces. We are looking forward to his significant contributions on this project.

We met in November with the Huntington group conducting the *in vivo* experiments. Samples are being shipped.

OBJECTIVES

Overall project objectives:

a) Selecting candidate surfaces that are likely to enhance the microscopic mechanical stabilization of a microstructure implanted within the central nervous system;

b) Selecting candidate organic surfaces that are likely to enhance the close approximation of neurons or neuronal processes to specific regions of implanted silicon microstructures;

c) Developing or adapting available methods to bond the selected organic molecules to a silicon dioxide surface like the surface of a micromachined electrode (Langhe and Wise, 1992) and to chemically characterize these surfaces before and after protein adsorption.

   1. The attachment method shall be stable in saline at 37°C for at least 3 months;

   2. To use silane coupling as the method of attachment;

   3. To use the silanes to control the spatial extent (i.e., the pattern) of the deposited surface.

d) Developing a cell culture or other suitable model of mammalian cortex and investigate the growth and adhesion of neurons, glia, micro-glia, and other cells present in the nervous system on substrates coated with the selected surfaces;

e) Cooperating with other investigators in the Neural Prosthesis Program by coating microelectrodes as required by over the contract period with the most

promising materials for *in vivo* evaluation as directed by the NINDS Project Officer.

FOURTH QUARTER OBJECTIVES

- Continue working to establish cortical cell culture conditions for 4 week survival
- Begin screening biologically modified SAM surfaces for cortical cell survival
- Continue screening E22 response to artificial surfaces
- Continue surface analysis of surfaces both before and after culture
- Continue surface stability experiments in saline + BSA at 37°C
- Finish screening surface for glial response to non-ideal SAM surfaces
- Continue screening non-ideal surfaces for microglia response
- Begin PN10 culture experiments
- Send more samples to Huntington to continue *in vivo* experiments

BACKGROUND

Biomaterials that penetrate into the central nervous system as the microscopic electrode shafts of neural prostheses interact with neural and other tissues on a cellular and molecular level. In order to achieve tight coupling between these implanted microelectrodes and the target neural substrate, this interaction must be understood and controlled. Controlling the interaction requires an understanding of how cells, including neurons and glia, and extracellular proteins respond to the surface chemistry and any leachable substances of implanted biomaterials. This contract supported research will study these interactions with a long-term goal of rationally designing microelectrode surfaces to promote specific tissue interactions.

Presently, available clinical neural prosthetic implants typically use stimulus levels that excite volumes of neural tissue ranging from cubic millimeters to cubic centimeters around the electrode. Because of the large stimulus intensities required, precise control of the response of neurons within the first few cell layers of an implanted electrode has not been necessary. Recent developments in the areas of micromachining and fabrication of silicon integrated circuit microelectrodes have introduced the possibility of controlled

stimulation of smaller volumes of neural tissue--on the order of one thousand to one hundred thousand times smaller than those used today.

The efficiency of these microelectrodes depends on the micro-environment around stimulating sites. The surface of the microelectrodes and the proteins that adsorb to this surface have a major impact on the way in which different cell populations react to the implant. Neural growth cones are sent out from many neurons around a microelectrode following implantation. With appropriate surfaces it may be possible to get selected neurons to send processes directly to the microelectrodes. Glia and other cells also respond to an implanted electrode. With appropriate surfaces it may be possible to promote cell adhesion and anchoring of some areas of the implant structure while leaving other areas with minimal response from glial cells. This study will investigate cellular and molecular responses to specific surface modifications of silicon microelectrodes.

RESULTS

Surface Analysis and Stability Measurements

X ray photoelectron spectroscopy (XPS) is necessary for this program in the same way that an NMR spectrometer is necessary for conducting an organic synthesis program. Since we are synthesizing surfaces and modifying their properties, we will need to assay the result of the surface before (starting material) and after (reaction product) modification. This is analogous to examining a procedure by NMR. One would not think to run an organic synthetic reaction with only an occasional examination of the actual product; in the same sense, it is crucial for us to examine the product in our surface modification experiments.

The stability of most of the surface monolayers on glass coverslips screened so far have been monitored by XPS for twelve weeks in PBS alone and in a 0.001% BSA solution in PBS at 37°C. As the data in Table I show for a 13 F monolayer, the slips in the PBS/BSA solution are rapidly coated with a thick protein layer from the BSA, as indicated by the increased C 1s intensities, while the slips in PBS alone show relatively minor increases in C, perhaps from adventitious contamination. The stability of the 13F is conveniently indicated by the F 1s intensity, which decreases for both PBS alone and for PBS/BSA.

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Table 1

Comparison of XPS intensities for 13F modified glass coverslips in PBS and PBS/BSA over time. Intensities were first normalized to a constant Si 2p intensity, then further normalized to the initial F 1s or C 1s intensity for the 13F surface before immersion in solution (time = 0).

Time(days)	F 1s		C 1s	
	PBS	PBS/BSA	PBS	PBS/BSA
0	1.00	1.00	1.00	1.00
1	--	0.80	--	0.96
3	--	0.70	--	4.60
7	0.10	0.60	1.30	5.80
14	0.10	0.40	0.80	3.90
28	0.00	0.40	1.60	5.70

However, the growth of the large protein overlayer in the case of PBS/BSA is responsible for most, if not all, of this decrease, because the thick overlayer attenuates the photoelectron flux from the 13F film below. In the case of PBS alone, the F 1s decreases must be attributed to the loss of 13F molecules from the film, probably by hydrolysis as has been reported by other workers. Thus, the data indicate that the deposition of protein onto the 13F monolayer from the PBS/BSA solution prevents hydrolysis and thereby enhance the stability of the film. This provides a basis for understanding the long-term effects of surface monolayers on cell culture systems, where proteins are part of the medium and are produced by the cells as well. These data sets, which include a wide range of surfaces utilized in this project, require further analysis for a detailed model of the structure of the films over time.

XPS analysis experimental conditions included an angular aperture of nominally 5° a spot size of 600μ in diameter, and a take-off angle of 35° from the surface plane unless otherwise noted. Experimental uncertainties are estimated to be 1-3 atomic % based on the capabilities of XPS, and are not based on statistical variations of a large number of trials.

Cell Culture

We established a rudimentary serum-free culture system for the cortical cells to more closely approximate the composition of cerebral spinal fluid (CSF). As the extension of time in culture is critical for comparing the development of different birthdays of neurons *in vitro*, one of our principle aims is to prolong survival and viability of cortical neurons in culture to 4 weeks. We concentrated on defining and examining the culture conditions to optimize longevity. In this capacity, we evaluated various tissue culture conditions (such as medium, additives, neurotrophic factors) which could play important roles in culture. We subsequently analyzed the impact of conditional manipulation and modifications on survival and cell type with immunohistochemical techniques and XPS.

We continued to evaluate various neurotrophic factors indicated by the literature to promote survival of cortical neurons in culture. We focused on BDNF which has been shown to promote survival of cortical neurons in culture (Ghosh, et al., 1994; Jones, et al., 1994), bFGF (reviewed by Tempe and Qian, 1995), NT-4 (Ip et al., 1993), GDNF (Tomac et al., 1995), and CNTF (Arakawa et al., 1990). We also examined the role that population density plays in neuronal survival in culture because other workers (Fukushima et al., 1995) have shown that increased neuronal plating density has significant impact on cell survival. Our preliminary results also support this finding. Cell counts were taken to determine survival at 24 hr. and 7 D in culture at two population densities (50 X 10³ cells vs. 100 X 10³ cells per cm²) in the presence of various neurotrophic factors. Two conditions are illustrated in Figures 1 and 2. In each case, the cell plated at the higher density suffered less mortality with time. Those cells plated on poly-lysine in the presence of CNTF suffered only 14% mortality, at high density, as compared with 52% mortality at the lower density. Controls suffered 28% mortality by Day 7 at high density, and 36% mortality at low density. The effect of the neurotrophic factor is less clear. While neurotrophic and factors have been shown to have a beneficial role, sometimes a they can function in a deleterious role, in cultured neuronal cells (Koh et al., 1995), and our results in the third quarterly report. Note that while CNTF seemed to provide a beneficial influence in one case, it appears to contribute to mortality at lower cell concentrations.

Like the preliminary results obtained above in the long term culture with different cell densities and neurotrophic factors, manipulation of culture medium and additives can have a significant impact on cell survival. These conditions can have disparate effects, as seen in this case, at different time points in development in culture. We examined cortical cells plated on poly-L-lysine (PL) in Neural Basal medium (NB), with or without B27 or N3 additives, at two timepoints, 5 and 11 days (Figure 3). These results, substantiated by cell counts, show that NB alone is the worst at both time points, but that the effects of the additives vary with time and culture. Initially, survival is less in NB + B27, and best in NB + N3; but at Day 11, the situation is reversed with NB + N3 no longer supporting survival.

Cell lineage studies suggest that there are two distinct types of macroglial cells that have been identified in the rat cerebral cortex: astrocytes and oligodendrocytes (Grove et al., 1993), and two types of neurons: pyramidal and non-pyramidal cells (Price and Thurlow, 1988; Parnavelas et al., 1991. In the cortex, pyramidal neurons use the excitatory transmitter glutamate, whereas most non-pyramidal neurons use the inhibitory neurotransmitter GABA (Peters and Jones, 1984; Houser et al., 1984; Jones and Hendy, 1986). The pyramidal cells are projection neurons and are most characteristic of the cortex and have the form of an isosceles triangle with the apical and basal dendrites. The axon emerges from the base of the cell and descends toward the brainstem and spinal cord. Non-pyramidal are interneurons with short processes. It is known that all the neurons and macroglial cells that comprise the adult cerebral cortex are derived from the embryonic precursor (neuroepithelial) cells in the ventricular zone. The initial phase of CNS developments is characterized by the proliferation of the precursor cells, followed by the generation of neurons and glia. The neurons are differentiated into different neurotransmitter phenotypes. Cell phenotype choice to become neurons or glia and neurotransmitter choice to become glutamatergic or GABAergic are crucial steps in cortical development. However, the factors that control the timely differentiation of the precursor cells into differentiated cells types are still mainly unknown. It is believed that the cell environment plays a key role in the specification of cortical cells, even though a cell intrinsic development program is important in regulating cell lineage (Williams and Price, 1995).

These cultures were examined up to 21 days in culture and then stained with glial astrocyte with mouse anti-glial fibrillary acidic protein (GFAP, Sigma) or neuronal cell markers, rabbit anti neuron-specific enolase (NSE, Chemicon). There was a considerable rise in the presence of glial astrocytes starting about day 14, becoming significant by Day 21 when the experiment was terminated, Figure 4. While many cells were found to be immunoreactive to GFAP, about 5% of cells stained with antiserum to neuron-specific enolase. There were also regions consisting solely of neuronal cells.

In the last report we showed preliminary data on astrocyte culture on various surfaces, those considered ideal (PEDA, DETA, PL) and some considered non-ideal (TP, 13F, OTS). We noted that surfaces that are initially the best gradually peak and decline, while some surfaces that are initially poor perform better over the longer term. This observation is critical as results after 24 hours or even 7 days are potentially not predictive for viability over longer time periods. There was overall a correlation with lower survival on non-ideal surfaces. Given our understandings of how certain neurotrophic factors affect neurons in culture, we were interested in examining how one of these, bFGF, would then affect survival of glial astrocytes. Our results strongly suggest that bFGF exerts protective effects on astrocytes in vitro. Results of study are included in a preparation of a manuscript.

Collaborations

We have established a collaboration with W. Agnew at the Huntington Medical Research Institute. While we have not yet specifically modified electrodes, we have successfully modified the poly-silastic sheath that holds a series of microelectrodes in place along the spinal cord during implantation (Agnew et al., 1990). One problem we are focused on solving is that the sheath has glial scar buildup and adhesions that eventually displace the electrodes laterally (with adhesion) and vertically (due to buildup of tissue). We visited Huntington Institute after the Neuroscience meeting in November, and have planned a series of experiments. These will be described in the next report. We are also examining by surface analysis some postmortem samples supplied by D. Agnew's group.

NEXT QUARTER OBJECTIVES

- Continue working to establish cortical cell culture conditions for optimal 4 week survival
- Begin screening biologically modified SAM surfaces for cortical cell survival
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EFFECTS OF PLATING DENSITY ON CELL SURVIVAL
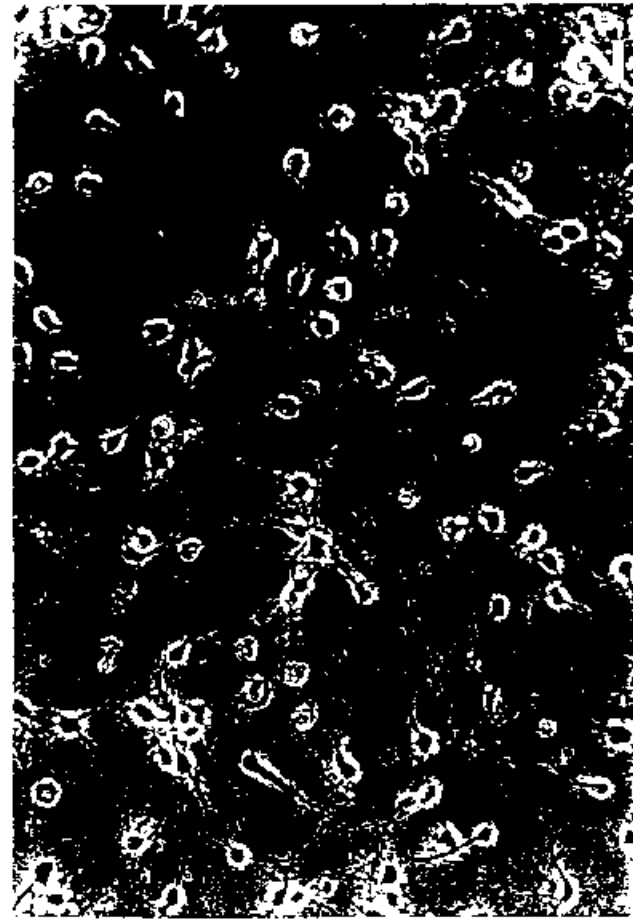
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Day 7, 50 X 10^3 / cm^2
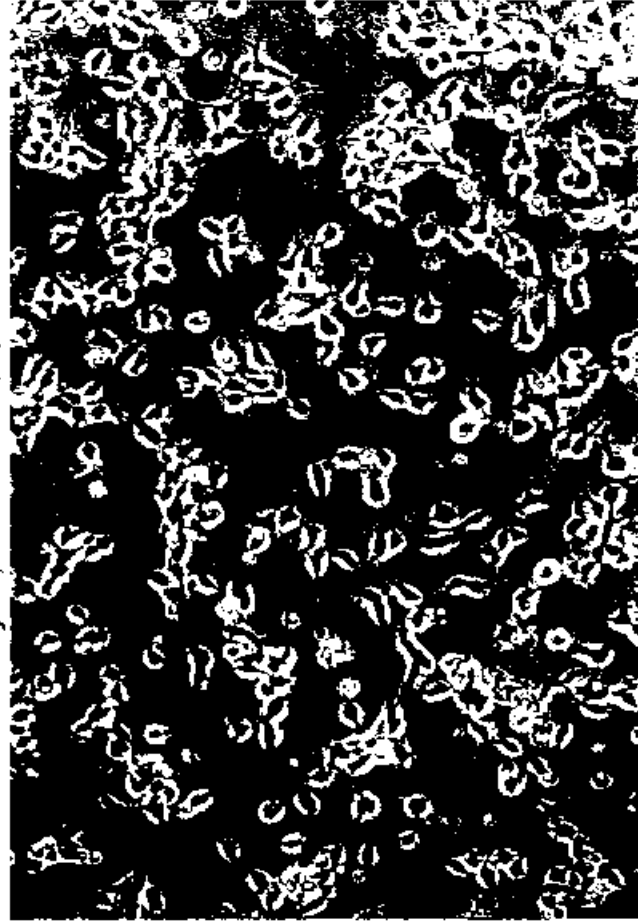
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EFFECTS OF PLATING DENSITY ON CELL SURVIVAL

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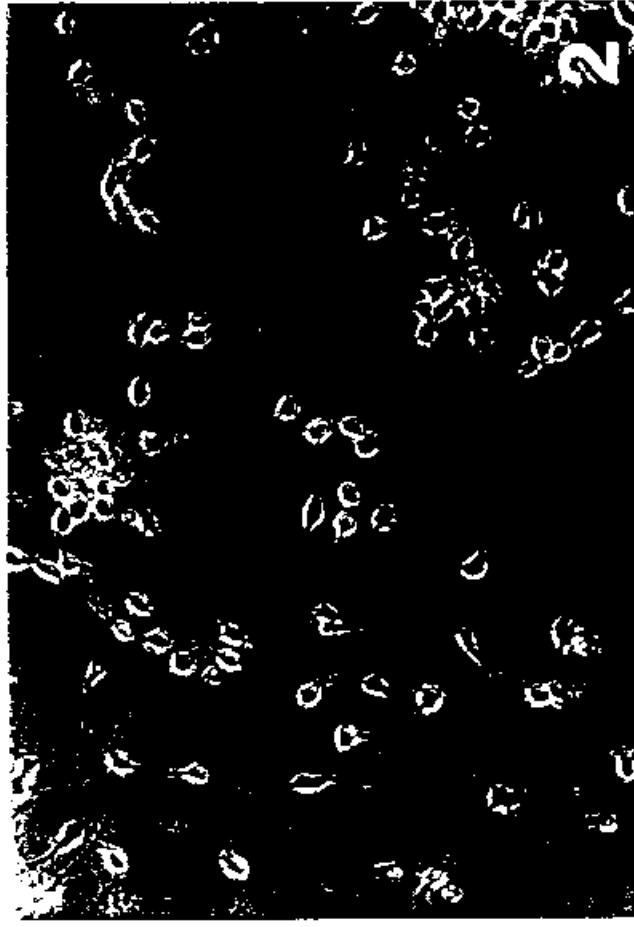
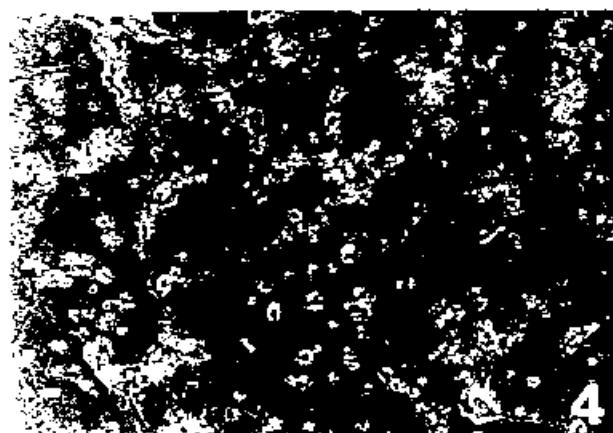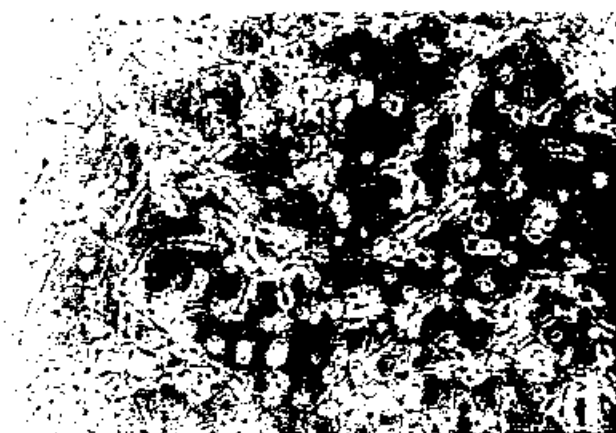Day 7, 50 X 10^3 / cm^2, CNTF
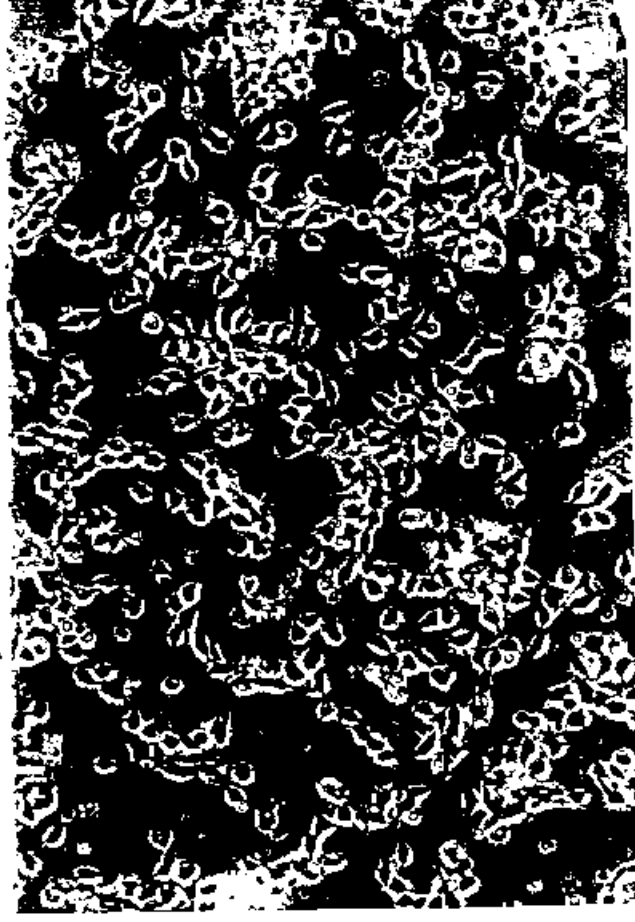
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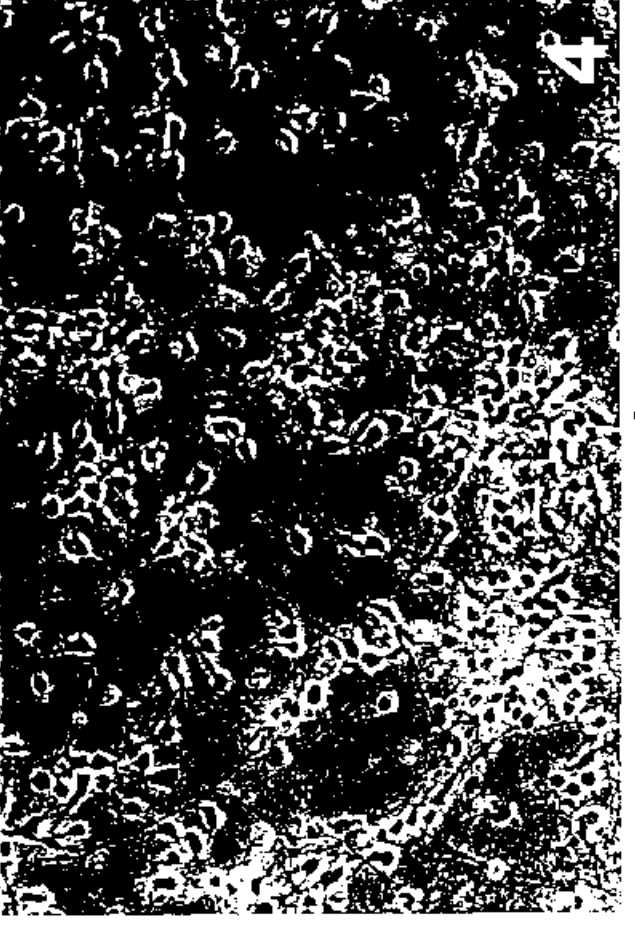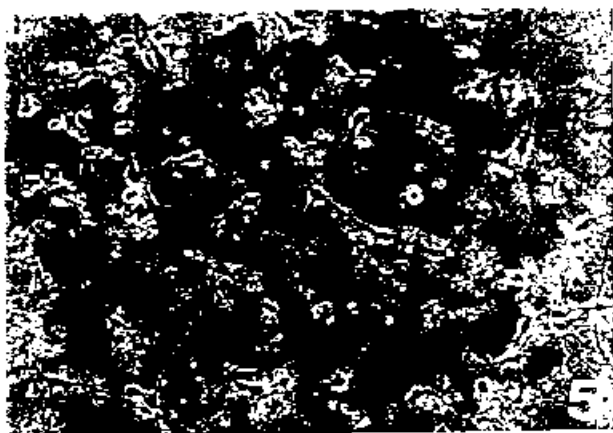
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CELL SURVIVAL IN NEUROBASAL/N3 OR NEUROBASAL/B27 MEDIUM

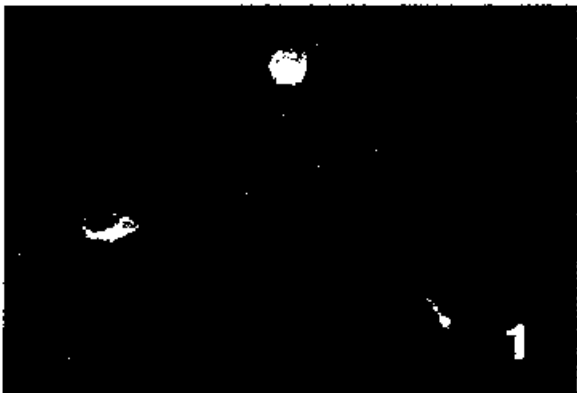5-day-old culture   11-day-old culture
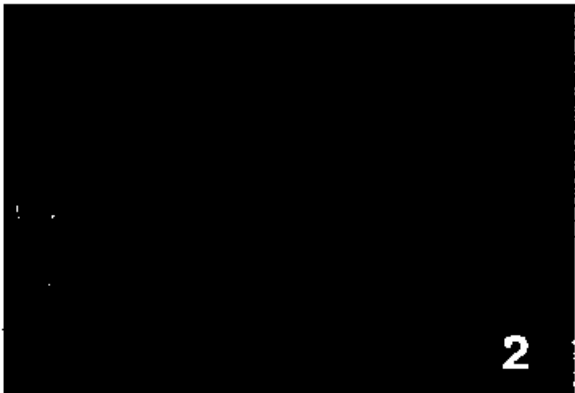

Neurobasal Medium

Neurobasal/N3 Medium
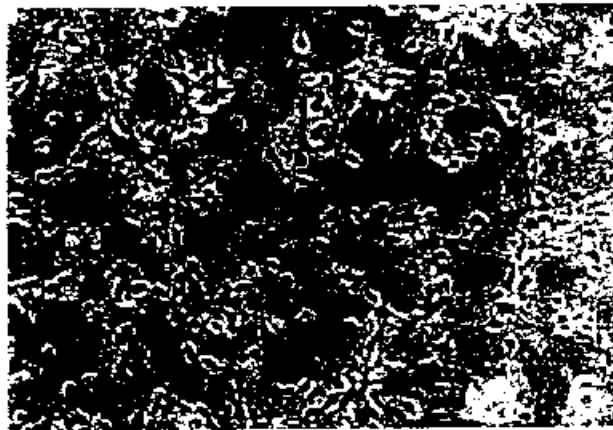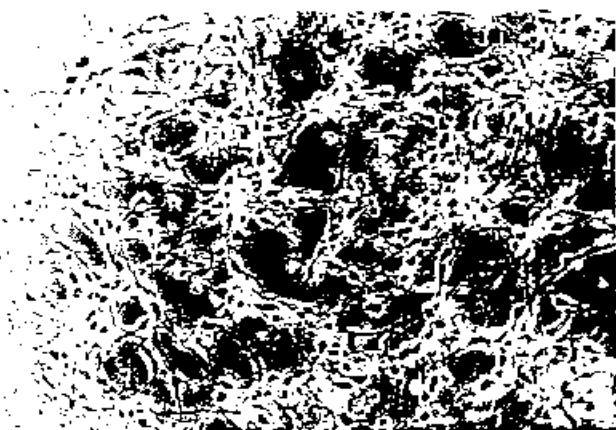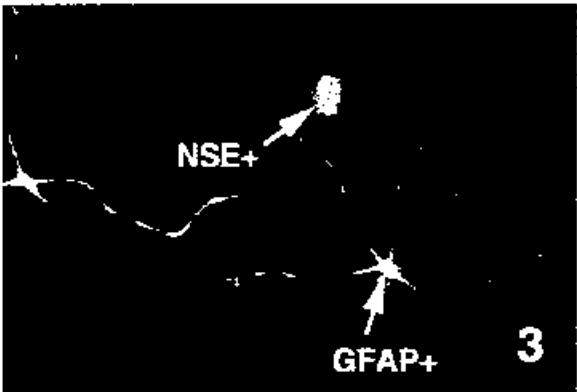
Neurobasal/B27 Medium

Survival of cerebral cortical cells in 21-day-old cultures

DETA

DETA

DETA + NT4

Poly-D-lysine + CNTF

Poly-D-lysine

Poly-D-lysine + CNTF